

# Facultative role for T cells in extrafollicular Toll-like receptor-dependent autoreactive B-cell responses in vivo

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Extrafollicular (EF) B-cell responses are increasingly being recognized as an alternative pathway of B-cell activation, particularly in autoimmunity. Critical cellular interactions required for the EF B-cell response are unclear. A key question in autoimmunity, in which Toll-like receptor (TLR) signals are costimulatory and could be sufficient for B-cell activation, is whether T cells are required for the response. This is pivotal, because autoreactive B cells are considered antigen-presenting cells for autoreactive T cells, but where such interactions occur has not been identified. Here, using AM14 site-directed transgenic rheumatoid factor (RF) mice, we report that B cells can be activated, differentiate, and isotype-switch independent of antigen-specific T-cell help,  $\alpha\beta$  T cells, CD40L signaling, and IL-21 signaling to B cells. However, T cells do dramatically enhance the response, and this occurs via CD40L and IL-21 signals. Surprisingly, the response is completely inducible T-cell costimulator ligand independent. These results establish that, although not required, T cells substantially amplify EF autoantibody production and thereby implicate T-independent autoreactive B cells as a potential vector for breaking T-cell tolerance. We suggest that these findings explain why autoreactivity first focuses on self-components for which B cells carry TLR ligands, because these will uniquely be able to activate B cells independently of T cells, with subsequent T–B interactions activating autoreactive T cells, resulting in chronic autoimmunity.

systemic lupus | autoantibodies

**A**lthough much attention has been recently focused on the germinal center (GC) reaction—which creates long-lived memory and plasma cells—some B-cell responses engage primarily the extrafollicular (EF) pathway of activation (1). This response occurs predominantly in the marginal sinus bridging channels at the border of the T-cell zone and red pulp, spilling into the adjacent red pulp areas. These locations are rich in dendritic cells and other myeloid cells but lack follicular dendritic cells. EF responses rapidly generate large numbers of short-lived plasmablasts. The location and cell types inherent in the EF response suggest that it may be regulated quite differently from the GC response and may generate different cellular products with different biological implications. The EF pathway is seen transiently in T cell-dependent responses to protein antigens (Ags) (1) and more recently has been appreciated as a major and sometimes sustained component of pathogen responses, particularly to bacteria (2, 3).

Despite its likely importance in immunity, many aspects of the EF response remain poorly defined. For example, the roles of T cells in the EF response are not clear. The EF response is thought to undergo minimal, if any, somatic hypermutation of Ig variable regions in responding cells and similarly to have little isotype switch (1). Mutation and switch are often thought to rely on T-cell signals, yet it remains poorly defined whether these processes are influenced by T cells in the EF response. In this connection, immunization with T-independent Ags induces

a robust but transient and largely unswitched EF response, suggesting that T cells are not required (4).

Recently, it has become apparent that autoantibody responses also proceed through the EF pathway (5, 6). Evidence for this comes from multiple mouse systems and can be inferred from the production of plasmablasts and short-lived autoantibody responses in humans (5, 7–9). It seems that a prominent requirement for systemic autoimmune EF responses is that the self-Ag contain a Toll-like receptor (TLR) ligand that is recognized by a B cell-intrinsic TLR. Indeed, targets of autoantibodies such as DNA, RNA, and self-IgG [in this case as immune complexes (ICs) with IgG antinuclear Abs] do contain TLR ligands. Conversely, when an artificial Ag is provided with a TLR9 ligand, it engages a predominantly EF response (10).

The identification of a TLR-driven EF-based activation of autoreactive B cells has broad implications for understanding the mechanisms that initiate and propagate B-cell autoimmunity, most of which have yet to be fully explored. For example, activation of autoreactive B cells at EF sites promotes disease by generating pathogenic autoantibodies as well as activated B cells that in turn can mediate damage by Ab-independent mechanisms, in particular by promoting autoreactive T-cell activation (11). Therefore, it is critical to understand the cellular interactions and signals that are required for and control EF responses in autoimmunity, because any of these could be therapeutic targets.

Given the pivotal nature of breaching T-cell tolerance in the establishment of chronic autoimmunity, it is important to determine whether EF responses in autoimmunity involve T cells, and if so, to understand how these interactions take place. We have been using AM14 B-cell receptor (BCR) transgenic (Tg) mice (AM14), specific for self-IgG2a of the “a” allotype [rheumatoid factor (RF) specificity], to study spontaneous and induced RF responses that both use the EF pathway (5, 12). AM14 B cells are activated by IgG2a antichromatin in vitro and in vivo in a TLR-dependent fashion (13, 14). In vitro and presumably in vivo the antichromatin Abs complex with ubiquitous chromatin released from dying cells, forming antigenic chromatin-containing ICs. In vivo, IgG2a antichromatin stimulates a robust EF response without evident GCs that depends on a combination of TLR7 and TLR9 and requires MyD88 expression in the B cell (14).

When studying only IgM responses in conventional IgM BCR Tg mice, we found that induced plasmablast responses were of normal magnitude in a T cell-deficient MRL/lpr background (14).

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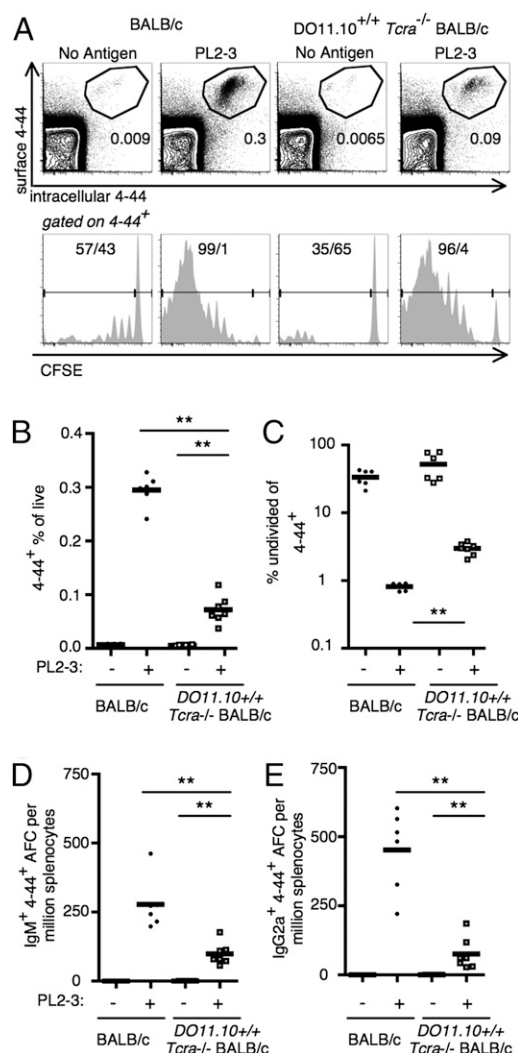
This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1018571108/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1018571108/-DCSupplemental).

However, larger numbers of somatic mutation in T-sufficient contexts suggested that T cells could influence the response. Nonetheless, direct evidence for T–B collaboration in this system was lacking, and the signals involved were unknown. To determine whether T cells do indeed affect the response, and if so how, we have used a new AM14 BCR site-directed (sd)-Tg mouse model, which can undergo isotype switch, as well as a BALB/c background to eliminate confounding influences of the autoimmune milieu (15). Using a combination of genetic and inhibitor approaches, we have found that the TLR-dependent EF autoantibody response in this model can indeed proceed to completion in the absence of T cells, including plasmablast differentiation and isotype switch. However, we also found that Ag-specific T cells can greatly augment the response. This occurs in a CD40L and IL-21-dependent fashion but is inducible T-cell costimulator ligand (ICOSL) independent. This indicates that the response can initiate without T cells but that the EF response is nonetheless an important site for T–B collaboration in the generation of classic autoantibodies. This has important implications for understanding how autoimmunity initiates, as well as for how to block it.

## Results

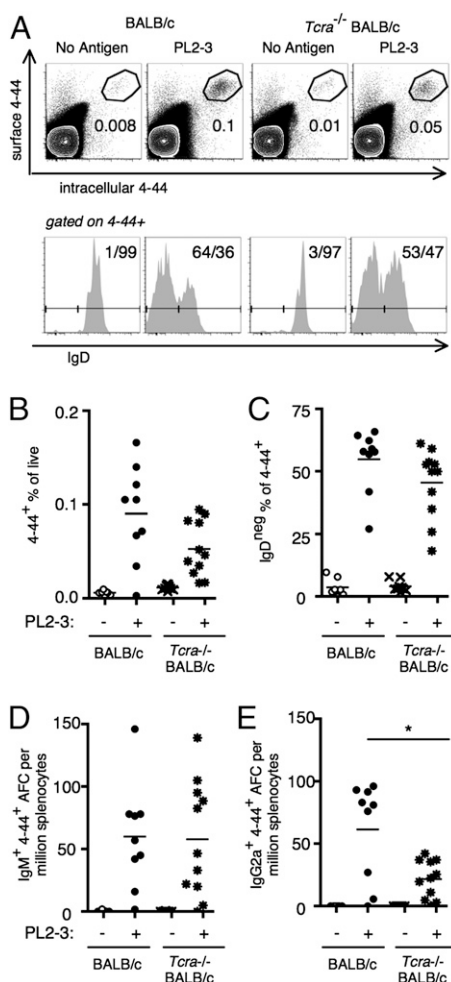
**Effects of T Cells Restricted to an Irrelevant TCR on the EF RF Response.** T cells can provide help to B cells in an Ag-specific or nonspecific manner (16). We tested the requirement for Ag-specific T-cell help by transferring AM14 sd-Tg BALB/c B cells into DO11.10<sup>+/+</sup> TCR $\alpha^{-/-}$  BALB/c mice. In these experiments, nearly all T cells in the mice were specific only for an ovalbumin peptide and thus should not be able to help AM14 B cells in their response to antichromatin Abs. The presence of T cells of an irrelevant specificity preserved lymphoid architecture and controlled for nonspecific effects of the absence of T cells. In the AM14 sd-Tg mice a small subset of B cells expressing certain V $\kappa$ 8 family L chains recognizes self-IgG2a, and we identify these cells using the 4-44 antiidiotype Ab (5, 17). In both DO11.10<sup>+/+</sup> TCR $\alpha^{-/-}$  BALB/c and WT recipients, the transferred 4-44<sup>+</sup> AM14 B-cell population expanded in response to the IgG2a antichromatin Ab, PL2-3, at day 6, as detected by flow cytometry, but not if Ag was not given. However, PL2-3-induced expansion of 4-44<sup>+</sup> AM14 B cells was decreased approximately fourfold in the DO11.10 BALB/c recipients compared with WT recipients (Fig. 1*A*, *Upper*, and 1*B*). Antigen-specific division of 4-44<sup>+</sup> cells in both DO11.10 and WT recipients in response to PL2-3 was detectable using dilution of carboxyfluorescein succinimidyl ester (CFSE). However, proliferation of 4-44<sup>+</sup> cells was decreased in DO11.10 recipients compared with WT recipients, as shown by an increased fraction of undivided 4-44<sup>+</sup> cells in the PL2-3-treated DO11.10 mice (Fig. 1*A*, *Lower*, and 1*C*). Similarly, both WT and DO11.10 recipients were permissive for 4-44<sup>+</sup> Ab-forming cell (AFC) differentiation. However, the generation of both IgM and IgG2a 4-44<sup>+</sup> AFC populations was reduced in DO11.10 recipients compared with WT recipients (Fig. 1*D* and *E*), with IgG2a being somewhat more affected than IgM. DO11.10 mice have a stable, albeit smaller, CD4 T-cell compartment (Fig. S1*A*). This provided normal lymphoid architecture in DO11.10 recipients to host EF plasmablast responses similar to those found in WT recipients (Fig. S1*B*). Thus, by all parameters analyzed, lack of specific T-cell help does not eliminate the ability of 4-44<sup>+</sup> cells to respond. However, specific T-cell help has a role in optimizing 4-44<sup>+</sup> B-cell proliferation and differentiation.

**Effects of T-Cell Deficiency on the EF RF Response.** Although indicating a role for Ag-specific T-cell help, the prior experiment does not eliminate the possibility that T cells could also be helping B cells to initiate the response in a nonspecific manner. If T cells also had a nonspecific role, elimination of all T cells would abrogate the response. To test this, we transferred AM14 sd-Tg BALB/c B cells into TCR $\alpha^{-/-}$  BALB/c mice, which lack all  $\alpha\beta$  T cells, or into control BALB/c mice. We then activated the



**Fig. 1.** Specific T-cell help is not required for but enhances the RF B-cell response to IgG2a antichromatin Abs. BALB/c or DO11.10 TCR Tg, TCR $\alpha$ -deficient BALB/c mice were killed on day 6, and splenocytes were analyzed after transfer of purified AM14 sd-Tg B cells and administration of PL2-3 as indicated in *Materials and Methods*. (*A*) Representative flow cytometry plots are shown for mice in treatment groups, as indicated for both rows. *Upper*: Live events. *Lower*: 4-44<sup>+</sup> events. (*A*, *Upper*, and *B*) 4-44<sup>+</sup> surface and intracellular double-positive cells were quantitated. (*A*, *Lower*, and *C*) Proliferation was assessed using CFSE dilution with 4-44<sup>+</sup>CFSE<sup>hi</sup> cells considered to be undivided. (*D* and *E*) Splenic 4-44<sup>+</sup> AFCs of IgM (*D*) or IgG2a (*E*) isotype. Data are compiled from two independent experiments. \*\**P* < 0.01, Mann-Whitney two-tailed test.

AM14 B cells with PL2-3 and analyzed the response at day 5. The expansion of 4-44<sup>+</sup> B cells was comparable in TCR $\alpha^{-/-}$  mice compared with WT controls (Fig. 2*A*, *Upper*, and 2*B*). Similarly, IgD down-regulation was comparable between the two groups of mice (Fig. 2*A*, *Lower*, and 2*C*). The 4-44<sup>+</sup> IgM AFC numbers were also not different between WT and TCR $\alpha$ -deficient mice (Fig. 2*D*). In contrast, numbers of 4-44<sup>+</sup> IgG2a AFCs were reduced  $\approx$ 2.5-fold in TCR $\alpha^{-/-}$  mice, although still substantial in quantity compared with the unimmunized TCR $\alpha^{-/-}$  controls (Fig. 2*E*). These results indicate that  $\alpha\beta$  T-cell help is not required for initiating the AM14 B-cell response to PL2-3, but such T cells optimize the differentiation of IgG2a AFCs. There is therefore no evidence of a nonspecific effect of T-cell help. If T cells were providing help nonspecifically, we would have found a lower response in the fully  $\alpha\beta$  T cell-deficient animals; if any-



**Fig. 2.**  $\alpha\beta$  T cells are not required for but enhance the RF B-cell response to IgG2a antichromatin Abs. BALB/c or TCR $\alpha$ -deficient BALB/c mice were killed on day 5, and splenocytes were analyzed after cell transfer of purified AM14 sd-Tg B cells and administration of PL2-3. (A) Representative flow cytometry plots are shown for treatment groups as indicated and are gated as described in Fig. 1A. (A, Upper, and B) 4-44<sup>+</sup> surface and intracellular double-positive cells. (A, Lower, and C) Fraction of 4-44<sup>+</sup> cells that had lost expression of IgD. (D and E) Splenic 4-44<sup>+</sup> AFCs of IgM (D) or IgG2a (E) isotype. Data are compiled from four independent experiments. \**P* < 0.05, Mann-Whitney two-tailed test.

thing, the response is more robust in the mice lacking such cells compared with the T cell-restricted animals.

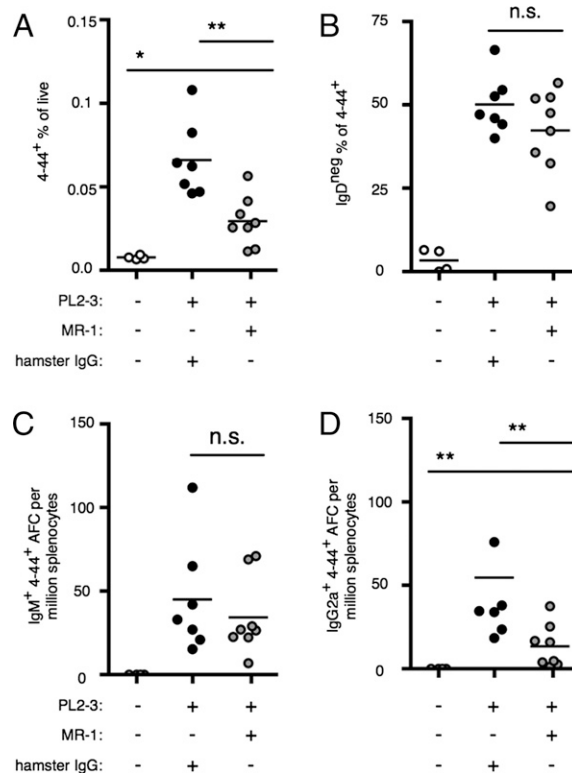
These results clearly show that Ag-specific T cells are not required to initiate the EF AM14 B-cell response to antichromatin Abs in vivo. Proliferation and differentiation were both observed in the absence of Ag-specific T-cell help. However, T cells do seem to have a role in supporting the amplification of the EF B-cell response because the response was quantitatively diminished in the absence of T cells. Further, we infer that Ag-specific T-cell help is required for an optimal response, because an environment containing nonspecific T-cell help (Fig. 1) did not augment the AM14 B-cell response at all compared with an environment lacking  $\alpha\beta$  T cells (Fig. 2). The magnitude of the control responses was somewhat smaller in these experiments compared with those in Fig. 1, which we attribute to a different lot of Ag as well as a 1-d difference in the duration of the response. It is not clear why the reduction of AM14 B-cell response to antichromatin antibodies in DO11.10 recipients was more robust compared with the reduction seen in  $\alpha\beta$  T cell-deficient mice.

DO11.10 mice contain T<sub>reg</sub> (Fig. S1A), and these may regulate the AM14 B-cell response in an Ag-nonspecific way.

**Molecular Mechanisms of T-Cell Help for the EF Response.** To investigate mechanisms by which Ag-specific T cells help EF B cells, we tested three components of T-cell help: CD40L signaling, IL-21 secretion, and ICOS signaling. All of these are known to be important for the generation and maintenance of the GC pathway (18, 19), but none has been tested in vivo in this type of autoreactive EF response.

To test the role of CD40L in PL2-3 induction of AM14 B-cell activation, we used the blocking Ab MR-1 (20), comparing it with a hamster IgG control. CD40L was blocked before and during PL2-3 activation of transferred AM14 sd-Tg BALB/c B cells in BALB/c mice, and the response was observed at day 5 after activation. Expansion of the 4-44<sup>+</sup> population was observed in both groups but was suboptimal in mice treated with MR-1 (Fig. 3A). Within the 4-44<sup>+</sup> population, a similar proportion of cells had down-regulated IgD in both groups (Fig. 3B). The 4-44<sup>+</sup> IgM AFC population was also comparable in both groups (Fig. 3C). In contrast, CD40L-blockade reduced the frequency of 4-44<sup>+</sup> IgG2a AFCs that developed in response to PL2-3 (Fig. 3D). Thus, CD40L interactions are important for optimizing the AM14 B-cell response to PL2-3.

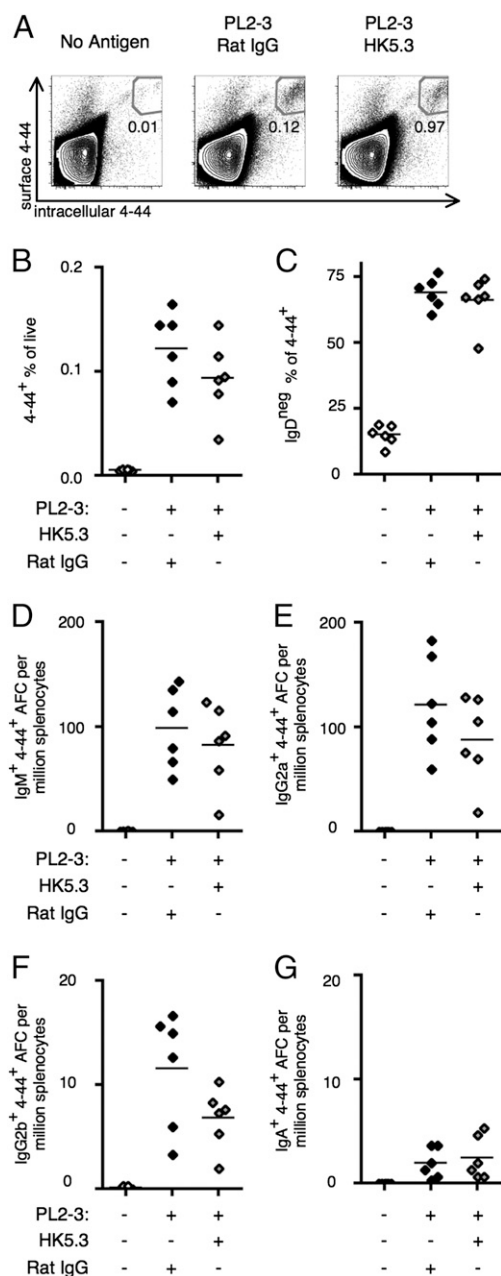
IL-21 secreted by T follicular helper (T<sub>FH</sub>) cells has been shown to support GC responses (18). Most pertinent to the AM14 response to antichromatin Ab, a subset of T cells that resembles T<sub>FH</sub> has been identified by FACS and histology to be located in the EF regions of diseased MRL/lpr and other autoimmune-prone mice (21). These EF T cells express ICOS and



**Fig. 3.** Blocking CD40L only partially inhibits the RF B-cell response to IgG2a antichromatin Abs. BALB/c mice were killed on day 5 after cell transfer with CD40L blocking and PL2-3 as indicated. (A) 4-44<sup>+</sup> surface and intracellular double-positive cells. (B) Fraction of 4-44<sup>+</sup> cells that had lost expression of IgD. (C and D) Splenic 4-44<sup>+</sup> AFCs of IgM (C) or IgG2a (D) isotype. Data are compiled from three independent experiments. \**P* < 0.05 and \*\**P* < 0.01, Mann-Whitney two-tailed test.







**Fig. 5.** Blocking ICOSL does not inhibit the RF B-cell response to IgG2a antichromatin Abs. BALB/c mice were killed on day 6 after transfer of AM14 sd-Tg B cells and administration of PL2-3 along with ICOSL blocking or control Ab. Representative flow cytometry plots are shown, gated as described in Fig. 1A. (A and B) 4-44<sup>+</sup> surface and intracellular double-positive cells. (C) Fraction of 4-44<sup>+</sup> cells that had lost expression of IgD. (D–G) Splenic 4-44<sup>+</sup> AFCs of IgM (D), IgG2a (E), IgG2b (F), or anti-IgA (G) isotype. Data are compiled from two independent experiments.

It seems likely that B cells activated by TLR ligand-containing self-Ag must interact with and activate T cells to fully expand and maintain the response. Once such a T–B collaborative amplifying loop is established, the response could become self-sustaining, leading to chronic autoimmunity (27).

The experiments described here provide important insights into the influence of T cells on the development of an autoreactive B-cell response. First, we found that specific T cells are required; AM14 B cells responded similarly whether T-cell help was of an irrelevant specificity or completely absent. Second, we

observed that CD40L is an important ligand in the process, further implicating T cells and strongly suggesting the need for a cognate interaction between T and B cells. Third, IL-21, although not essential—as with other T-cell signals—influences the response in both quantitative and qualitative fashions (promoting IgG2a and IgG3 switching) via its direct action on responding B cells. IL-21 has previously been implicated in contributing to murine models of autoimmune disease, presumably via multiple effects on T and B cells (28); here we define a specific influence directly on the autoreactive B cell. Taken together, we conclude that Ag-specific T cells optimize the AM14 B-cell response to PL2-3 in vivo through CD40L and IL-21 but are not required for either initiation or completion of differentiation to the plasmablast stage (Fig. S4).

This work also provides an in vivo link to T EF helper cells first characterized by Odegard et al. (21) that produce IL-21 and can promote plasmablast expansion and switching in vitro. Although ICOS is required for the development of T EF helper cells in chronically autoimmune mice, our data support an ICOS-independent mechanism in helping B cells during their initial activation and differentiation. This supports earlier work implicating a requirement for ICOS in B-cell responses to protein Ag but not polysaccharide Ag (29). Our data support the idea that the requirements for effective T-cell help adapt depending on the nature and the persistence of the Ag. In systemic autoimmunity, Ag is constitutively generated from dying cells. This may allow synergy of BCR with TLR signaling to bypass the requirement for ICOS.

The requirement for specific T cells does raise an important unanswered question: what is the nature of the auto-Ag(s) for these T cells? There is no obvious foreign Ag being introduced when PL2-3, a syngeneic IgG2a purified from syngeneic animals, is introduced. Presumably T cells are activated that recognize self-Ag in the chromatin-containing IC and/or the self-IgG. Despite expectations of self-tolerance, several groups have shown that chromatin or spliceosome protein-specific T cells do exist in the periphery of normal animals and that they can be activated, particularly when specific B cells present Ags (30, 31). Similarly, T cells specific for self-IgG have been described (32). It will be of great interest to identify the specificities and types of T cells that are providing help to the pathogenic EF response.

In general, the emphasis on “T-dependent” vs. “T-independent” responses in classical model systems may have clouded the issue of what happens in a more physiological setting with biologically relevant Ags. In our case, we have used an Ag relevant to the production of both RF and antichromatin Abs in vivo. This Ag has some characteristics of a T-independent type 1 Ag, with a TLR ligand and possibly an enhanced ability to crosslink the BCR given the multivalent nature of ICs and also of chromatin itself (14). Notably, pathogens, such as bacteria and viruses, would share many of these properties, although whether they are able to activate B cells in an analogous way remains unclear (33). With such Ags, the simple concepts of “T-dependent” or “T-independent” are inadequate to describe the in vivo interactions that control the EF B-cell response, and this is where the present work provides important insights. Our data demonstrate that all aspects of the response can and do happen in the absence of T cells, including expansion, differentiation, and isotype switch [mutation was also previously demonstrated (14)]. The significant implication of this observation is that self-reactive B cells can be activated all of the way to effector function without the need to break T-cell tolerance. We propose, therefore, that expression of nucleic acid-specific TLRs by B cells is a weak link in the systems that prevent autoreactivity but has been evolved as a result of the benefit from enhanced responses to microbial pathogens. Self-Ags that provide a TLR ligand will have a differentially greater ability to activate B cells in the absence of T-cell help, and this program of activation leads much further than previously thought. We suggest that this TLR requirement explains why antinuclear Abs are a final common outcome of many immune dysregulatory disorders, and

indeed, the EF pathway of activation is being identified in a variety of systems with B-cell autoimmunity (8, 27, 34).

## Materials and Methods

**Mice.** AM14 sd-Tg BALB/c mice were generated as previously described (15). BALB/c mice were purchased from Jackson Laboratories. DO11.10 Tg TCR $\alpha^{-/-}$  and TCR $\alpha^{-/-}$  BALB/c mice were obtained from Dr. Kim Bottomly (Yale University, New Haven, CT) (35). IL-21R $^{-/-}$  BALB/c mice (36) were obtained from Dr. Warren Leonard (National Institutes of Health, Bethesda, MD). CD45.1 BALB/c mice were obtained from Dr. Hyam Levitsky (Johns Hopkins School of Medicine, Baltimore, MD). All mice were maintained at Yale University School of Medicine under specific pathogen free conditions and used according to Institutional Animal Care and Use Committee-approved protocols.

**B-Cell Isolation and Adoptive Transfer.** Splenic B cells were prepared using the EasySep Mouse B Cell Enrichment kit (Stemcell Technologies) according to the manufacturer's instructions, resulting in purity of 95% or greater as determined by FACS, unless otherwise indicated. Three million B cells in PBS were injected i.v. per mouse, unless otherwise indicated.

**Ascites Preparation and Immunization.** PL2-3 hybridoma was grown and Ab was obtained as previously described (12). PL2-3 (500  $\mu$ g) was administered to animals i.p. on days 0, 2, and 4.

**CFSE Labeling.** Primary cells were cultured at 37° for 10 min in the dark with 10  $\mu$ M CFSE (Invitrogen) in PBS with 0.1% BSA.

**Blocking Antibodies.** To block CD40L, MR-1 mAb was purified from serum-free tissue culture supernatant using Protein G sepharose (Pharmacia) as pre-

viously described (17). Two hundred micrograms in sterile PBS was injected i.p. on days -1 and 2. To block ICOSL, 200  $\mu$ g HK5.3 mAb (22) in sterile PBS was injected i.p. on days 2 and 4.

**Flow Cytometry.** Cell preparation and staining was performed as previously described (17). Dead cells were excluded using ethidium monoazide staining. Abs were made in the laboratory as previously described (17) or purchased from vendors as indicated. The following staining reagents were used for these experiments: 4-44 (AM14 anti-Id)-biotin, 4-44 Alexa-647, anti-CD4 Pacific Blue (GK1.5), anti-CD22 PE (Cy34.1; BD), anti-CD25 PE (7D4; Southern Biotech), anti-CD44 Alexa 488 (IM7), anti-CD45 Alexa 488 (6B2), anti-CD95 PE (Jo2; BD), anti-FoxP3 APC (FJK-16s; eBioscience), anti-IgD FITC (11-26c.2a; Biologend), anti-TCR  $\beta$  biotin (H57-597; BD), PNA biotin (Vector Laboratories), and streptavidin (SA) PE-Cy7 (BD).

Samples were analyzed on an LSRII (BD). Data were analyzed with FlowJo software.

**ELISpot Assay.** ELISpot assay was performed and analyzed as previously described (12). Goat anti-Ig isotype coating Abs were purchased from Southern Biotech.

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